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Antimicrobial effect of *pleomeleangustifolia* pheophytin A activation with diode laser to *streptococcus mutans*

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Abstract. The main purpose of this research is to identify potential of Pheophytin A. as photosensitizer a agent to inactivate *Streptococcus muttans* using laser diode of 405nm. Pheophytina is known as chlorophyll derivate that losses magnesium ion at the center of porphyrin ring structure. In this research, phrophytin was extracted from Suji leaf (Pleomeleangustifolia). To determine the antimicrobial effect of treatments on *S.mutans*, samples were divided into three groups as follows: (1) Groups A(treated with Pheophytin A. and laser 405 nm at varying energy density of 2.5; 5, 7.5; 10.0; 12.5; 15.0; 17.5 and 20.0 J/cm^2), (2) Group C$^-$(negative control, no treated), (3) Group C$^+$ (treated only with pheophytin). The experiments were repeated at least three times for each group. The results were analyzed using analysis of variance and the Tukey test. A P value $\leq$0.05 was considered to indicate a statistically significant difference. The decrement of percentage of number of bacterial colonyes growth was defined as: $|(\Sigma \text{ sample colony} - \Sigma \text{ control colony})/\Sigma \text{ control colony}| \times 100\%$. The result showed that the incubation of Pheophytin A. using irradiation from laser diode of 405nm have a significant effect towards the decrement in bacterial growth. The most decreased percentage colony of *S. mutans* occurred on the incubation of pheophytin a treatment and laser irradiation 405nm with density 20 J/cm^2 is 61.9%. This showed that pheophytin a functions as a photosesitizer activator to inactivate *S. mutans* bacteria.

1. Introduction

Dental caries is a progressive pathological process of teeth's destruction, which is caused by combinations of diet, host, micro flora and exposure time [1]. Micro flora such as *Streptococcus mutans* is an acidogenic bacteria that colonized at teeth surface. Exposure time is the duration of teeth exposition towards acid produced by bacterial which causes teeth plaque. The main bacterial causing dental caries is a group of streptococci mainly *S. mutans*. *S. mutans,* which is a normal flora in oral. However, *S. mutans* might increase significantly at favorable environment and change to be pathologic [2].

aPDT is a non-antibiotic approach that was developed to inactivate microorganism, and it is a potential alternative compared conventional antibiotic [3]. aPDT combines a non-toxic photosensitizer and visible light resulting singlet oxygen and free radical that caused microbial cell destruction [4]. The main target of aPDT is an external microbial structure. Suitable adhesivity at bacterial structure causes destruction activated by light. Photosensitizer doesn't need to get into inside of the microorganism, therefore no resistance of microorganism [5].

Photosensitizer used in aPDT must have photophysical, photochemical, and photobiology characteristics to work optimally [6]. The basic structure of ensitizer consists of tricyclic dying with different mesoatom (acridine, proflavine, riboflavin, metilen blue, flurescein and eritrosin), tetrapyrolle (porphyrin and its derivate, chlorophyll, phylloerythrin and phthalocyanines and Furocoumarins (Psoralen and its methoxy derivate, xanthotoxin and bergaptene) [7].

Porphyrin is a class of aromatic heterocyclic that can be found easily in nature. Porphyrin has important biochemical process that functions including transportation (haem) and photosynthesis (chlorophyll). Because of its physical and chemical properties, porphyrin is used in various application such as artificial photosynthesis, oxidation catalysis, sensor, non-linear optic, microorganism photo in activation, and nanomaterial for cancer PDT [8]. Each of porphyrin molecule has the ability to absorb light at the specific wavelength. Light exposure at proper dose with wavelength spectrum corresponds to photosensitizer absorption spectrum of porphyrin, resulting photo inactivation of bacterial cell [9]. Photosensitization depends on the type and quantity of porphyrin and suitability between light spectrum and photosensitizer absorption spectrum [10].

Chlorophyll molecule consists of a porphyrin (head structure) having polar properties and a fitol in the tail. Porphyrin is formed from tetrapyrolle ring with a magnesium ion at the centre of tetrapyrolle ring. Phorphyrin ring in chlorophyll rules as light absorber (photosensitizer), therefore chlorophyll and its derivates are usefull as photosensitizer agents [11]. Pheophytina is a derivate of chlorophyll that lost magnesium ion at the centre of porphyrin ring structure. Pheophytin A. have two maximum wavelength absorptions in soret band area (408,9 nm) and Q band area (665,4 nm) [12].

According to previously research, diode laser is used as light source in photodynamic therapy because of the small beam, focused beam and high coherence. Finally, the purpose of this research is to explore the potential Pheophytin A. of Suji leaves (Pleomele angustifolia) as derivative chlorophyll and agent of photosensitizer to the process of inactivation of *S. Mutans* with laser diodes 405 nm.

2. Methods

2.1. Bacterial strain and culture conditions

The sample strains used on this research was pure culture bacteria from *Streptococcus mutans*. The bacteria were collected from tooth of patients diagnosed with dental caries in Dental Hospital Airlangga University Surabaya Indonesia.

2.2. Materials

Pheophytin A. was extracted from Suji leaves (*Pleomeleangustifolia*). Figure.1 showed the character of Pheophytin A.. A standard solution 1mg/ml was made by using Dimethylsulfoxide (DMSO). Pheophytin A. solution with concentration 20% was made inside sodium hydrochloride (NaCl).

2.3. Light source

Laser irradiations were carried out using diode lasers with wavelength output of 405.52±0,23nm. The power outputs were 49,50±0.20mW with wide size: 0.39±0.01 cm^2.

2.4. Antimicrobial effect of treatments against a. actinomycetemcomitans with total plate count (TPC)

To determine the antimicrobial effect of treatments on *A. actinomycetemcomitans*, samples were distributed to 3 groups as follow: (1) Groups A(treated with Pheophytin A. and laser 405 nm at varying energy density of 2.5; 5, 7.5; 10.0; 12.5; 15.0; 17.5 and 20.0 J/cm^2), (2) Group C^-(negative control, not treated), (3) Group C^+ (treated with pheophytin only). The experiments were repeated at least three times for each group. The suspension was planted on Tryptocase Soy Agar (TSA) (Merck, Darmstadt, Germany) sterile media and incubated at a temperature of 37°C for 24 hours. After incubation, the number of colony-forming units per milliliter (CFU/ml) was determined. The results were analyzed by analysis of variance and the Tukey test. A P value ≤0.05 was considered to indicate

a statistically significant difference. The percentage decrease in the number of bacterial colonies growth defined as: | (Σ sample colony - Σ control colony)/ Σ control colony | x 100%.

3. Results and discussion

Pheophytin A. that used in this research was extracted from Suji leafs (*Pleomeleangustifolia*). The result of characterization Pheophytin A. using UV-Visible Spectroscopy is presented on figure.1.

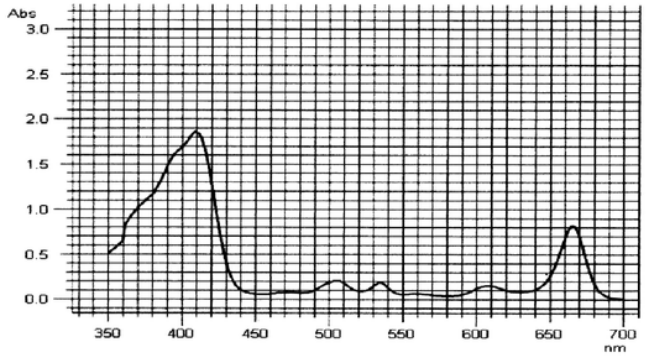

Figure 1. Spectrum Profile of Pheophytin A. from *Pleomeleangustifolia* Leaves

This research used a Pheophytin A. as a photosensitizer to inactivate *S. mutans* bacteri using laser diode 405nm. Pheophytin A. is a derivative from chlorophyll that has an ion magnesium loss in the center of porphyrin. Chlorophyll is a green pigment that can be found in every greenery that has a role in photosynthesis. *Pleomeleangustifolia* leaf, which is known as Suji leaf or *Pandan Betawi*, contains much chlorophylls that is commonly used in Indonesian society as a natural food coloring. The photosensitizer was Pheophytin A. solution with concentration 20% in NaCl 0.9% from standard pheophytin solution 1mg/ml DMSO. Before the treatment, absorption test of Pheophytin A. was done by using UV-Vis spectrophotometer to obtain the percentage of absorption on Pheophytin A. 20% solution. Figure.1 shows that maximum absorption of soret band and Q band located at 405 nm and 665 nm corresponds to literature from Masami kobayaki *et.al.*, 2013 located at 408.9 nm and 665.4 nm. It showed that profile spectra Pheophytin A. of *Pleomeleangustifolia* leaf has a similar characteristic of maximum absorption of soret band and Q band similarly to the previous research.

The absorbance of Pheophytin A.. at laser diode 405nm is equal to log (1/transmittance). Percentage of Pheophytin A. absorption is (1-transmittance) x 100%. Result measurement of absorbance of Pheophytin A. used UV-VIS Spectroscopy at spectrum 405nm is 2.3082. Percentage of Pheophytin A. absorption is 99.5%. The dose that was capture by Pheophytin A. laser diode can be calculated by Dose = (output power/wide beam) x irradiation time x percentage of Pheophytin A. absorption at 405nm. The dose that were (2.5; 5, 7.5; 10.0; 12.5; 15.0; 17.5 and 20.0) J/cm^2.

3.1. Photoinactivation study of s. Mutans incubated Pheophytin A. using 405 nm diode laser

Instruments diode laser light source was used in this study. Performance spectrum laser test showed peak spectrum at 405.52±0,23nm. The output power at 49,50±0.20 mW with broad beam 0.39±0.01 cm^2. The measurement of output power at 2 cm distance showed that the stability power up to measurement of 1000 seconds. The measurement of temperature during exposure to the sample showed the elevated temperature within the range of bacteria *S.mutans* growth. Thus, the death of bacteria were actually caused by laser irradiation treatment not the influence of the environment.

Result of the effect of laser irradiation of various energy laser 405nm with photosensitizer Pheophytin A. was examined by using ANOVA Factorial Test. Table 1 shows summary of the result of ANOVA Factorial Test.

Table 1. Analysis result of treatment of laser diose 405nm towards *S. mutans* bacteria that were incubated with Pheophytin A.

Group	Dose (J/cm^2)	N	% *S. mutans* colonyes reduction		Factorial anova
			Average	SD	significantly
Laser	2.5^{a}	4	0.3	0.0	P=0
	5.0^{b}	4	4.1	0.4	
	7.5^{bc}	4	5.7	0.6	
	10.0^{d}	4	9.1	0.6	
	12.5^{de}	4	10.9	1.2	
	15.0^{ef}	4	12.3	1.2	
	17.5^{fg}	4	13.8	1.1	
	20.0^{fg}	4	14.0	0.6	
Laser with Pheophytin A.	2.5^{c}	4	6.5	0.5	P=0
	5.0^{g}	4	15.3	1.1	
	7.5^{g}	4	15.2	0.3	
	10.0^{h}	4	25.6	0.4	
	12.5^{i}	4	45.0	0.9	
	15.0^{j}	4	42.9	0.6	
	17.5^{k}	4	57.9	0.7	
	20.0^{l}	4	61.9	0.6	
	Total	64	21.3	19.1	

Description: N = sample size. SD = standard deviation. The same superscript showed no significant difference from the results of the Tukey test.

Result of this test showed that laser irradiation, with incubation of Phoephytin A. or not, effected the reduction of amount of colonies of bacteria *S.mutans*. Multi comparison test used was *Post Hoc Multiple Comparison Tukey* to show and analyse the difference group of treatments.

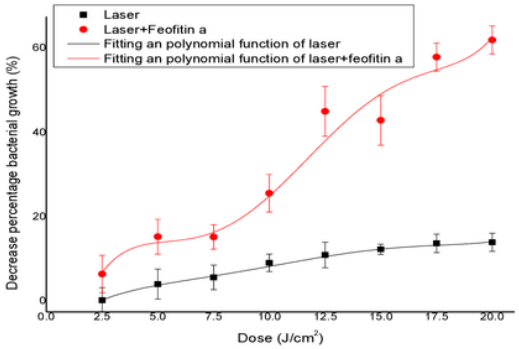

Figure 2. The result of laser diode 405 nm treatment of *S. mutans* with and without Pheophytin A.

Figure 2 shows polynomial graph of the result of bacteria *S.mutans* treatment of laser diode 405nm with Pheophytin A. (R^2=0.92) and without Pheophytin A. (R^2=0.99). Percentage of the decrement of *S.*

mutans colonies that were incubated with Pheophytin A. with laser irradiation 405nm and energy 5.0 and 7.5 J/cm^2 proportional trough the decrement by using energy 17.5 J/cm^2 and 20.0 J/cm^2.

The result of ANOVA test of the treatment of *S. mutans* group that was incubated with Pheophytin A. and laser irradiation showed significant differences toward decrement percentage of colony bacteria *S. mutans* with treated only using laser. The dose increase from 10 J/cm^2 until 20 J/cm^2.

The most significant decrement of percentage colony *S. mutans* toward the incubation of Pheophytin A. treatment and laser irradiation 405nm with energy 20 J/cm^2 was 61.9%. It showed that Pheophytin A. Functions as a photosensitizer activator to inactivate *S.mutan* bacteria.

4. Conclusion

The result of this research showed that incubation of Pheophytin A. with irradiation of laser diode 405nm has a significant effect toward bacterial growth. The most significant decrement of percentage colony of *S. mutans* toward the incubation of Pheophytin A. treatment and laser irradiation 405nm with energy 20 J/cm^2 was 61.9%. It showed that Pheophytin A. functions as a photosensitizer activator to inactivate *S.mutan* bacteria.

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